The Use of Flow Cytometric DNA Ploidy Analysis As an Adjunct to Detection of Minimal Residual Disease in B-lineage Acute Lymphoblastic Leukemia

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DNA Ploidy Analysis
for the Detection of Minimal Residual Disease
in Acute Lymphoblastic Leukemia

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The detection of minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL) has clear prognostic implications, as MRD-positivity during or after induction chemotherapy is associated with poor outcome and increased risk of leukemic relapse. However, the detection of MRD in B-lineage ALL by flow cytometric immunophenotyping can be difficult in the post-therapy bone marrow, due to an increase in normal B-cell precursors that can be confused with leukemic blasts. The aim of this study was to assess whether flow cytometric DNA ploidy analysis, in tandem with flow cytometric immunophenotyping, can be used as a sensitive means of detecting residual or relapsed ALL in patients with previously documented aneuploid cell populations. We retrospectively studied all cases of ALL at our institution over a 12 year period from 1991-2003 (n=114). Aneuploid clonal populations were present in 32% of patients (n=37). Of this group, 24 had "normal" immunophenotypes, as defined by phenotypic similarity of the leukemic clone with normal precursor B-cells, and 13 had "aberrant" immunophenotypes predominantly manifest as simultaneous expression of myeloid markers. Aneuploidy detected the presence of residual or relapsed disease in all cases where disease was found by flow immunophenotyping (normal n=8; aberrant n=7). In the group with normal immunophenotype, aneuploidy detected post-remission disease in three patients and MRD in one patient in whom the diagnosis could not be made with confidence by immunophenotyping. In the aberrant group, aneuploidy detected MRD in two patients in whom immunophenotyping failed to show positivity, likely because of downregulation of myeloid antigens on leukemic blasts. These results suggest that flow cytometric DNA ploidy analysis may be a useful and sensitive adjunct in determining relapse or presence of MRD in patients with B-lineage ALL.

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INTRODUCTION

General Introduction

Acute Leukemias are the leading cause of cancer death in the United States among individuals under 35 years of age. Affecting over 4000 people each year, and comprising more than 23% of all cancers in children under age fifteen, Acute Lymphoblastic Leukemia (ALL) in particular is a significant oncologic threat.[1-2] With the evolution of hematology and the advent of molecular diagnostics and flow cytometric immunophenotyping, diagnosis and classification of the acute leukemias has become a detailed and precise endeavor. This diagnostic revolution has not spared ALL, in which T- and B-cell lineage, clonality, stage of maturation, DNA content, cytogenetic abnormality, and specific immunophenotype can be determined rapidly and reliably using modern laboratory methods. Precise diagnosis has been paralleled by the formulation of effective treatment regimens, leading to an impressive success rate in achieving complete clinical remission using modern multi-agent chemotherapeutic protocols. Improved therapy has resulted in the redefinition of ALL from a nearly incurable disease to one with a 5-year survival rate of up to 80% for children younger than 15 years of age.[3-5] However, the fact remains that approximately 25 to 30% of children and 70% of adults with ALL will experience a relapse of their disease.[6-8] With this issue arises the need for early detection of recurrence and prognostic or risk-stratification based on the presence of post-therapy residual leukemia.

As the sensitivity of laboratory techniques has escalated, the concept of minimal residual disease (MRD) has emerged. In general terms, MRD is the presence of leukemia in a specimen the analysis of which by morphologic means alone would not demonstrate disease. The detection of MRD has been shown to be a significant prognostic factor in the outcome of patients with ALL.[6,8-16] The presence of MRD at various intervals during and after induction chemotherapy predicts relapse, and multiple studies have found that outcome is quantitatively related to the level of MRD at a given time interval.[8-10] This prognostic power is sustained even when controlling for other known prognostic variables, and it has been suggested that MRD levels may be useful for risk-stratification of patients into more or less intensive treatment groups when used as an indicator of response to therapy. Such risk-grouping could potentially save rapid responders from chemotherapy-associated morbidity and mortality while simultaneously giving poor responders a better chance at remission with intensified therapy.[12,14]

Multiple techniques have been employed to detect MRD in ALL, beginning with light microscopy of bone marrow specimens, cytogenetics, and fluorescence in-situ hybridization (FISH) against leukemia-associated genetic targets. With time, significantly more sensitive methods have been developed to detect residual leukemic clonal populations, including flow cytometric immunophenotyping and polymerase chain reaction (PCR) amplification of antigen receptor genes or chromosomal translocations. These more recently developed techniques provide sensitivities several orders of magnitude greater than that of traditional methods.[17] Despite their enormous power, the optimal use of these techniques is not completely established, and each has its own strengths and limitations. Flow cytometric assays maintain the advantages of rapidity, cost-effectiveness, and freedom from dependence on pre-identified genetic alterations in the leukemic clone. Additionally, the literature has clearly shown flow cytometric immunophenotyping of post-remission bone marrow specimens to be predictive of outcome in ALL.[6,14] Therefore, many institutions, including our own, choose to rely on this technology. However, flow cytometry has its disadvantages as well. One problem of interest is that detection of MRD can be obscured by the presence of normal precursor B-cells (hematogones), which are often present in increased numbers in post-therapeutic bone marrow.[18-19] This issue becomes especially salient when the leukemic clone does not possess a unique and distinguishing immunophenotype. The decreased specificity of immunophenotyping in this circumstance implies the need for an adjunctive diagnostic criterion in order to maintain analytical power.

DNA ploidy of leukemic cells has historically been a well-recognized prognostic factor in childhood ALL. Hyperdiploid karyotypes, defined by the presence of 51 to 65 chromosomes or a DNA index (DI) of 1.16 to 1.6, are associated with an improved outcome.[20-21] Conversely, hypodiploid karyotypes with less than 46 chromosomes are harbingers of a poor outcome.[22-24] Consequently, flow cytometric methods are employed to measure the DI of diagnostic marrow specimens in order to identify aneuploid cell populations for the purposes of prognostication. However, the use of flow cytometric DNA ploidy studies for the detection of recurrent disease or MRD in ALL has not been explored in the literature. In patients with a documented aneuploid leukemic cell population at diagnosis, marrow analysis for DNA ploidy may provide a sensitive means of detecting the re-emergence or persistence of a leukemic clone. This would provide especially useful information for patients with B-lineage ALL clones that happen to

lack aberrant immunophenotypes. A combined approach of ploidy and immunophenotypic studies has been shown to be effective in detecting MRD in one study of patients with multiple myeloma.[25] With a significant number of ALL patients harboring aneuploid cell populations at diagnosis as well, this method may be applicable to many leukemia patients and provide a useful adjunct in the detection of MRD.

ALL epidemiology

Acute lymphoblastic leukemia is the leading cause of cancer death in the United States among young people. With an incidence of 1.3 per 10^5 persons, it is the culprit in 23% of all childhood cancers and in 75% of childhood leukemias. ALL is the most common cancer in children aged 0 to 14, although the proportion of cases drops significantly between ages 15 and 19. According to most authors, peak incidence occurs between ages 2 and 3. However, there is a second smaller peak in incidence occurring in the seventh decade of life.[1-2] These statistics have changed somewhat with time, from an incidence of 27.2 cases per million in 1975-1979 to 34.5 cases per million in 1995-1997. The greatest proportion of this increase occurred between 1975 and 1984, although the reason for this is not well understood.[2]

In terms of demographic factors, ALL has an increased incidence among male rather than female children, with the greatest difference apparent during the pubertal period. Race also appears to play a role, with nearly twice the rate of ALL among white children when compared to the African American population.[26] The absolute difference in incidence between African American and Caucasian children is 15.2 vs. 27.8 per million, and this asymmetry appears to be due mainly to a 2.4-fold increased rate of ALL in white children between ages 0 and 4.[2] Nationality also appears to correlate with ALL incidence independent of race, with developing countries exhibiting lower rates of ALL when compared with developed nations. Interestingly, most developing nations that have been studied demonstrate an incidence similar to that of the United States in the mid- to late-1970's.[27] Again, the cause of this incidence shift is not known, but multiple theories have been posited, with the most attention given to issues of chemical and pollutant exposure.

ALL biology

Acute lymphoblastic leukemia is believed to arise in the setting of malignant transformation of a single lymphoid progenitor. As is the case with most malignant neoplasia, it is likely that transition to uncontrolled growth begins with multiple sequential genetic changes. Spontaneous mutations or leukemogenic translocations conspire to dysregulate the cell cycle, leading to unlimited expansion capability and the capacity for indefinite self-renewal.[28-30] The events associated with mutation and clonal expansion can precede the onset of clinical disease by years, and in the case of infantile ALL, it has been shown that leukemogenesis may even have its origins in-utero.[31]

Leukemic blasts in ALL usually show fidelity to one hematopoietic lineage by phenotypic classification. However, the extent of maturation within this lineage may vary widely, which suggests that leukemogenic transformation results from aberrant regulation of the normal marrow differentiation process. This can explain the heterogeneity of the disease, as it may arise during any point along the pathway of lymphoid differentiation. Once induced into malignant transformation, leukemic blasts become locked into their discreet stage of development. At this point, the proliferating progenitor serves as a template for clonal expansion. Indeed, it has been shown that ALL is a clonal process, as evidenced by the consistency of chromosomal translocations and immunophenotype across blasts within a given patient. However, the best evidence for ALL clonality has been divined through X-chromosome inactivation studies, which have shown consistently that blasts within a given clone maintain the same pattern of X-inactivation.[32]

Somatic mutations of the lymphoid progenitor can lead to expansion and longevity by several mechanisms, including increased proliferative rate, decreased apoptosis, or the expression of telomerase.[33] These mutations may arise de novo in any child, but they are seen at higher rates in some populations. Notably, children with constitutional chromosomal abnormalities are at increased risk for ALL. For example, children with the characteristic trisomy 21 of Down's Syndrome experience a 15-fold increase in the risk of leukemia, predominantly ALL.[34] Additionally, children affected by disorders characterized by chromosomal fragility, such as Bloom Syndrome or Fanconi's Anemia, are at increased risk. Even without identifiable constitutional anomalies or disorders, there is an elevated risk of leukemia in family members of leukemia patients. It was shown by several authors in the 1970's that siblings of leukemic children are generally considered to have a 2- to 4-fold risk of developing leukemia in their lifetime relative to the

general population.[35-36] However, these situations do not explain the majority of leukemia incidence. A more generally applicable explanation for the evolution of leukemia centers on the fact that normal lymphoid development is itself a high-risk endeavor. It is likely that there is an increased chance of spontaneous somatic mutation during the natural, and normally well-regulated, process of lymphoid gene-rearrangement in the bone marrow. This elevated degree of genetic shuffling, coupled with the high rate of lymphoid proliferation particularly in the developing marrow of young people, may set the stage for leukemogenic transformation.

Approximately 80 to 85% of ALL patients have leukemic phenotypes corresponding to B-lineage progenitors.[37-40] True B-cell ALL is rare, accounting for 2-3% of cases, but B-progenitor ALL is common and predominates over T-cell ALL. Occasionally, clones will demonstrate bi-phenotypic differentiation, with both T- and B-lineage immunophenotype, or expression of myeloid markers may be evident. Some patients also present with bi-clonal leukemias, wherein more than one clonal expansion has taken place. However, the majority of cases of ALL fall within the category of immature B-lineage disease. Within the B-progenitor group, different levels of maturation can be identified, leading to classification as pre-B, marked by expression of cytoplasmic immunoglobulin mu heavy-chains, and early pre-B, which lacks this expression pattern.[30] Immunophenotyping can accurately delineate lymphoid from myeloid, B- from T-lineage, and subsequently classify maturational stage by expression patterns of CD markers. However, morphology is also used to classify ALL. The French-American-British (FAB) classification for B-lineage ALL is well established and fairly simple. There are three classes defined by this working group, designated as L1, L2, and L3. L1 morphology represents 85% of cases; L2 composes 14% of cases and is more common among adult patients; and L3 is rare, making up 1% of cases and carrying the worst prognosis.[41] This system is now used in conjunction with molecular and immunologic studies.

Genetic anomalies associated with ALL are common and include aberrancies in both chromosomal number and structure. Structural gene rearrangements are quite common in ALL. Translocations are found in up to 75% of cases and are usually associated with activation of cellular oncogenes.[42] Examples in B-lineage ALL include activation of *myc* by t(8;14) translocations, E2A-PBX-1 by t(1;19), E2A-HLF by t(17;19), MLL-AF4 by t(4;11), MLL-ENL by t(11;19), *bcr-abl* by t(9;22), and TEL-AML1 by t(12;21).[30,43] There are many more examples in T-cell ALL. These rearrangements

typically dysregulate the function of a gene that is responsible for cell cycle control and lead to uncontrolled cellular proliferation. Deletions of tumor suppressor genes, such as *p53* and *p16* have also been documented in many cases of ALL. Alterations in chromosomal number are somewhat less prevalent, but are still fairly common with a role in between 20 and 40% of ALL cases.[44-46] Aneuploidy can manifest as either hypo- or hyper-diploid cell populations, depending on whether or not DNA content is reduced or increased respectively. Hyperdiploidy is frequently due to trisomies, most often of chromosomes 4, 6, 10, 14, 17, 18, 21, and X.[2] Abnormal ploidy often has clinical and prognostic significance (see below).

ALL diagnosis

The clinical presentation of patients with ALL is largely determined by the degree of bone marrow infiltration and/or extramedullary spread of leukemic cells.[1-2,30] Unfortunately, presenting symptoms can be quite non-specific. Historical and physical findings that raise suspicion include pallor, fatigue, bone pain, petechiae or purpura, bleeding, or fever. These findings generally represent a failure of normal hematopoiesis, although the addition of lymphadenopathy, hepatomegally, and/or splenomegally may indicate extramedullary spread. Symptoms attributable to anemia (Hgb<10g/dL) are present in roughly 80% of patients presenting with ALL, and those related to thrombocytopenia (Plt<100,000 per μL) are seen in up to 75%.[2] Indicators of extrameduallary spread are quite common as well, with hepatosplenomegally seen in roughly 65% of patients, and lymphadenopathy found in up to 50%. Less common sites of extramedullary disease at diagnosis include the CNS and testes, present in 5% of children and 10 to 15% of boys.[30]

The non-specific nature of many of these findings leads to a broad differential diagnosis, including atypical lymphocytosis associated with infectious mononucleosis or pertussis, ITP, CMV, EBV, or other pediatric malignancies with potential marrow involvement, including neuroblastoma, rhabdomyosarcoma, retinoblastoma, and non-Hodgkin's lymphoma. One additional, albeit less common, mimic is a left-shifted marrow in recovery from a previous toxic insult.[47] Most patients have been sick for days to weeks at presentation, and onset of symptoms can be either gradual or quite rapid.

Once suspicion has been raised through historical or physical findings, diagnostic testing is the next logical step. More than 90% of patients with ALL have clinically evident hematological abnormalities

evident at diagnosis.[30,48] As mentioned above, routine complete blood count (CBC) will show a hemoglobin of less than 10mg/dL in roughly 80% of patients, and platelet counts below $100x10^3$ per μL are nearly as common. The leukocyte count is, however, less predictable. Approximately 50% of patients present with elevated leukocyte counts greater than $10x10^3$ per μL, and 20% will have severely elevated counts above $50x10^3$ per μL. Still others will present with neutropenia or pancytopenia. Occasionally patients present in hyperleukocytosis syndrome, with counts exceeding $200x10^3$ per μL, leading to circulatory impairment and a more acute and varied clinical picture that requires emergent treatment.[47] A peripheral smear is indicated at the time of diagnosis in order to potentially identify circulating blasts in the leukemic patient. The vast majority of samples will exhibit identifiable lymphoblasts, but up to 10% of patients present with "aleukemic leukemia" and demonstrate no blasts in the peripheral circulation.[47]

Bone marrow aspiration is essential for the definitive diagnosis of ALL. Greater than 5% lymphoblasts in the marrow is highly suggestive of ALL, but most institutions require a 20 to 25% blast constituency in order to confirm the diagnosis.[2] In point of fact, about 75% of patients with ALL have greater than 50% marrow lymphoblasts at diagnosis.[30] In order to further classify and specify the nature of the blasts, cells from the marrow aspirate may be immunophenotyped by flow cytometry and sent for cytogenetic analysis.

The testing outlined above defines the principle diagnostic procedure necessary to confirm leukemia, and eventually the specific diagnosis of ALL. However, other testing will sometimes reveal or support identification of the leukemic process indirectly. Elevated serum uric acid secondary to increased cell turnover is sometimes noted in patients with ALL, particularly those with a very high leukemic burden. Serum lactate dehydrogenase (LDH) may also be elevated due either to increased cell lysis, ineffective hematopoiesis, or liver infiltration by leukemic cells. In addition to these abnormalities, a general metabolic dysregulation may occur, leading usually to either increased or decreased serum calcium levels and/or increased serum phosphate and potassium.[2]

Those patients suffering from extramedullary disease at presentation may warrant additional studies, including imaging of CNS or mediastinal mass lesions or bilateral testicular wedge biopsies. In every patient, regardless of evidence of an extramedullary process, it is advisable to perform a lumbar puncture with subsequent cytologic study of the CSF. In some cases, lymphoblasts may be evident in

significant numbers within the CSF, indicating the need for specific CNS-focused adjunctive therapy during treatment.

ALL treatment

Over the last several decades ALL has been transformed from a nearly uniformly fatal disease to one with an impressively favorable prognosis. This evolution has been due in part to two developing strategies in oncology, namely risk-group stratification and the use of intensive multi-agent chemotherapeutic regimens, as well as the ALL-specific precautions of CNS-preventive therapy and extended maintenance protocols.[2] Current therapy has four fundamental components. The first phase is induction, which is conducted with the goal of eliminating any evidence of leukemia by physical examination, laboratory values, and marrow examination. This equates to normal CBC and peripheral smear, lack of physical findings, and a marrow with normal cellularity and less than 5% blasts. In the pediatric population, remission can be induced in between 95 to 99% of patients.[49-50] The numbers are less impressive for adults, but remission is still achievable in between 75 and 90% of cases.[51] Along with this, CNS preventive treatment (or prophylaxis) is necessary using intrathecal chemotherapy and/or craniospinal irradiation.

The ability to induce remission in such a high proportion of patients is quite impressive. However, it is notable that without any post-remission therapy most patients will relapse within a median time-frame of only 1 to 2 months.[2] Without CNS preventive therapy this statistic is even worse due to CNS relapse. If disease recurrence is to be prevented, post-induction treatment must be instituted. The principle behind such therapy is to suppress further proliferation of the leukemic clone, to further reduce the circulating level of blasts, and to do so without allowing the evolution of drug-resistance. The first step towards these goals consists of consolidation therapy, which is an intensified treatment period following induction that is especially useful for high-risk patients. Consolidation serves as a "second hit" to the leukemic cell population after it has already been severely weakened. Even with the second hit, leukemic cells have shown to be resilient, and thus a prolonged period of maintenance therapy, up to 3 years in the case of ALL, has proven to be warranted.[52] The less intensive maintenance period subsequently functions in long-term suppression of the leukemic clone, either eliminating it completely, or more realistically

subduing it to the extent that it becomes inactive or is able to be managed by host immune responses.[49] Finally, supportive care throughout the treatment process is essential, including the use of blood components, treatment of infection, attention to metabolic and nutritional issues, and psychosocial support.

ALL relapse

In spite of the advances in treatment and the increased success of ALL chemotherapeutics, up to 30% of children, and a significantly greater number of adults, will relapse.[8,12] The marrow space is the most common site of relapse and defines the principal form of treatment failure in ALL.[2] Testicular relapse is less frequent, and rates have declined from 10 to 15% in the 1970's to close to 2 to 5% currently.[34, 53-55] Rates of CNS relapse have declined with effective prophylaxis, but this site remains a significant cause of treatment failure at just under 10%.[56-57]

With a new round of chemotherapy, reinduction rates of up to 90% have been achieved for those with marrow relapse.[58-59] The highest success rates are attained using new agents or combinations novel to the patient such that any drug-resistance can be overwhelmed. Other factors at play possess some prognostic value. A low WBC count at initial diagnosis or relapse and age between 2 and 10 years at diagnosis are features that predict successful reinduction.[60] Additionally, the length of the first relapse is correlated directly with the duration of the second remission.[61] However, with or without good prognostic features, the rate of reinduction unfortunately declines with each successive relapse.[2] At this stage the possible need for bone marrow transplantation becomes more salient, although its success is limited in this setting, and it can be logistically quite challenging.

ALL mortality and prognosis

Prior to the formulation of modern chemotherapeutic regimens, ALL was considered nearly incurable, with most patients surviving only 2 to 3 months from diagnosis.[30] In the 1960's, the 5 year survival rate for children aged 0 to 14 was essentially zero. However, by the late 1980's to mid 1990's the 5 year survival jumped to more than 80%. As mentioned above, it is thought that these improvements derived mainly from the implementation of risk-stratification and appropriately tailored multi-agent chemotherapy coupled with CNS prophylaxis and an extended maintenance period. Unfortunately, adult

ALL patients have not fared as well. In spite of a relatively high rate of remission induction, 5 year survival estimates for adults range from only 20 to 35%.[30]

There are multiple prognostic factors in ALL. Traditionally, the two most reliable and well-established criteria have been age and WBC count at diagnosis.[62] Patients less than 2 or older than 10 years of age have a poor prognosis relative to the interim years. The worst prognosis within this group is for infants less than one year of age, whose disease tends to be quite relentless.[63-64] Adults also do not fare well. The degree of initial leukocytosis has been cited by some as the single most important prognostic factor in ALL. There appears to be a linear relationship between WBC count and outcome in some studies, and with a count above $50x10^3$ per μL patients have a particularly poor prognosis.[65-66]

Other factors also appear to influence outcome. Boys have a worse prognosis in general than girls. In terms of race, African American children have a somewhat worse outcome than white and Hispanic children, who have roughly equivalent outcomes.[67] Additional factors, more biologic than epidemiologic, have been brought to light as genetic and molecular tests have evolved. There is an inverse correlation between prognosis and lymphoblast proliferation rate as measured by cytokinetic studies, and it is surmised that these kinetic factors may underlie clinical responsiveness.[30,68-69] Chromosomal abnormalities also carry prognostic significance in ALL. Translocations frequently underlie leukemogenesis. Many specific translocations have been identified, but some have been associated with outcome in a significant way. In particular, t(8;14), t(9;22), t(4;11), and t(1;19) predispose to early treatment failure, while t(12,21) imparts the chance of an improved outcome.[2] Aberrations in chromosomal numeracy also affect outcome. The clearest example of this is the association between hyperdiploidy, with a DNA index greater than 1.16, and improved prognosis.[20-21,44] Several reports have actually shown lymphoblast ploidy to be the most significant prognostic factor in childhood B-progenitor ALL.[45-46] It is thought that the benefits of hyperdiploidy may be related to increased leukemic sensitivity to antimetabolite chemotherapeutic agents.[70-71] Patients with Trisomies 4 and 10 also have an improved outcome.[72] Hypodiploidy, on the other hand, is a negative prognostic factor, with the worst outcome reserved for those with near-haploid genotypes.[22-24,73]

Immunophenotypic factors include lineage and maturation, with T-cell and mature B-cell ALL carrying worse prognoses than precursor-B-cell ALL. A somewhat more controversial idea is that aberrant

expression of myeloid markers may be associated with poor outcome. Finally, L3 morphology has been shown to be associated with a worse prognosis than either the L1 or L2 subtypes.[30] Of greatest interest to the topic of this report, minimal residual disease (MRD) has emerged in recent years as a major prognostic issue, and this will be visited in depth in the following section.

MRD background

Traditionally it was assumed that curing ALL was synonymous with elimination of all leukemic cells.[2] However, as sensitive means of detecting small numbers of cells in marrow samples have evolved, so has the realization that leukemic cells can remain in their host even after successful chemotherapeutic interventions. According to current standards, ALL is considered to be in remission when the blast population falls below the limit of morphological detection and constitutes less than 5% of non-erythroid marrow cellularity.[15,74-75] However, with a relatively standard leukemic burden of 10^{12} neoplastic cells at diagnosis, a patient in remission may continue to carry up to 10^{10} cancer cells.[12,20] Thus, a very successful several log reduction in leukemic burden may leave behind billions of clonal cells which may or may not at some stage repopulate the marrow in the form of relapsed disease. Although some have questioned whether or not these "remaining" cells constitute the vestige of a pre-leukemic clone or whether they indeed represent the original clone at diagnosis, it is fairly clear that the presence of minimal residual disease (MRD) may imply the presence of a leukemic stem cell capable of inciting recurrent disease.[17,49]

Detecting post-therapy residual disease began with simple morphologic examination of the bone marrow for visible blasts, allowing for a discriminatory sensitivity down to 5% neoplastic cells. This was later supported with the use of fluorescence microscopy and labeled polyclonal antibodies, initially limited to the study of T-cell ALL. With the advent of monoclonal antibodies and the isolation of B-lineage surface markers, this technology became more widely applicable to the majority of ALL cases. However, these techniques had limited sensitivity because they involved analysis of only small samples of marrow. A similar lack of sensitivity hinders fluorescence in-situ hybridization (FISH) studies and traditional karyotyping. The development of flow cytometry and its application to analysis of large numbers of cells in hematological malignancies allowed sensitivity to be increased significantly. In addition, PCR

technology became available in the 1980's and was applied to the problem.[76] These most recent methods provide greatly increased sensitivity for MRD detection.

MRD methods of detection

There are several requisites for creating a reliable and accurate assay for the detection of MRD. First, specificity is necessary in order to discriminate between normal and neoplastic cells. Second, sensitivity is needed to detect a relatively small number of neoplastic cells against a background of normal marrow constituents. Third, the assay must be standardized and reproducible. And, fourth, turnaround time from aspirate or biopsy to diagnostic report must be rapid enough to be clinically useful.[8]

However, difficulties exist in determining whether or not a patient with ALL is in "complete remission". First, there is massive sampling error attributable to the bone marrow biopsy or aspiration procedure. The percentage of marrow taken for examination is miniscule, and in the post-therapeutic marrow the number of blasts is greatly reduced. In addition, malignant cells may not be uniformly distributed within the medullary space. Secondly, the morphologic identification of MRD is of limited sensitivity. The lowest documented limit of detection by experienced cytologists was shown to be roughly 1%, and a limit of 5% is the more generally accepted number.[8,17] This is further compromised in the post-therapeutic marrow due to the presence of an increased number of normal hematopoietic precursors, or hematogones, that can be present at proportions greater than 5% and which can be quite easily mislabeled as blasts.[18-19] It has, unfortunately, been estimated that the amount of MRD detectable by microscopic morphology is 300-fold that required to cause relapse, and rough quantitation reveals that cytomorphological techniques may only be able to identify fewer than 30% of ALL patients who are destined to relapse.[8-9] Although occasionally useful for closer examination of morphologically suspicious cells, the additional methods of conventional karyotyping and FISH are also limited in sensitivity and cannot reliably detect sub-microscopic disease below 1 to 5%.[17]

The most widely accepted high-sensitivity methods in use today are flow cytometric immunophenotyping and polymerase chain reaction (PCR) gene amplification. Both of these methods, when applied meticulously, can achieve a level of sensitivity more than 100-fold greater than that provided by standard morphologic studies.[9,13-14,17] With this level of detection, submicroscopic residual disease can

be identified, and a more strict definition of remission may be cultivated. PCR technology involves the use of sequence-specific primers under changing temperature conditions in order to replicate DNA or mRNA sequences present in a sample. These sequences are amplified in logrhythmic fashion until the product is detectable on gel electrophoresis, whereupon its presence can be confirmed by both its position within the gel field and with radio-labeled probes to the sequence of interest.[16] Flow cytometric immunophenotyping utilizes analysis of light emission and scatter from monoclonal antibody-bound fluorochromes that attach to specific cell surface or intracytoplasmic markers. Data derived from this process allows demarcation of different cell populations by immunophenotype. Both of these processes can be directed towards the rather sensitive detection of leukemia-specific targets and therefore allow detection of MRD.

MRD methods of detection: PCR

There are two discreet classes of target for PCR amplification in the detection of MRD in ALL. One set of targets consists of the breakpoint fusion regions of chromosomal translocations. Because of the fact that breakpoint fusion regions associated with most known leukemic translocations are spread over large expanses within each gene locus, reverse transcription PCR (RT-PCR) of mRNA transcripts of these regions is more widely applicable than standard PCR. Being that the sequences of these transcripts are relatively stable across most patients who possess the same translocation, it is relatively simple to amplify and detect these targets without the need for customization for each patient.[77] The second set of targets for PCR are the functional regions of rearranged immunoglobulin heavy chain (Igh) or T-cell receptor (TCR) genes.[17] These rearrangements are unique to a given leukemic clone and will be present in every leukemic cell derived from it.

It has been found that MRD detection is highly concordant between these two types of targets.[10] However, each has significant positive and negative attributes. RT-PCR of fusion transcripts has the advantage of high sensitivity and relative rapidity of processing. In addition, translocations almost invariably remain stable through the course of the disease and are not easily influenced by clonal evolution. The ability to use prefabricated primers once a given translocation has been identified provides both resource and time savings for the diagnostic department involved. Perhaps the biggest drawback to using fusion transcripts is that specific chromosomal changes associated with well-defined breakpoint fusion

regions are present in less than 50% of ALL cases.[78-79] This imposes a serious restriction on the widespread use of such techniques, with applicability to roughly 40-50% of patients. Additionally, it can be difficult, or at least theoretically ambiguous, to quantify MRD using RT-PCR, as there may be variable expression of any given mRNA transcript depending on proximity to treatment, leukemic cell death, and other unpredictable parameters.[79] Beyond this, false negative results can arise due to mRNA degradation, and false positive results can arise in the setting of RNA cross-contamination.[17] Standard PCR of Igh and TCR rearrangements also has the benefit of high sensitivity, but can deliver extremely wide applicability due to the fact that virtually all ALL clones have a characteristic gene rearrangement. Applicability of this technique approaches 90% of ALL patients. In addition, quantitation of MRD can be achieved using these targets. The focus for this technique is amplification of DNA rather than mRNA transcripts; therefore there are a fixed number of targets to be amplified in each cell which do not vary with altered expression patterns.[17] The amplification product is truly based on the number of clonal cells present rather than on the genes they may or may not be expressing. Because Igh and TCR gene rearrangements are unique to each clone and therefore to each patient as well, a major drawback is the requirement that rearranged gene sequences must be identified at diagnosis in every ALL patient. After identification and sequencing, patient-specific junction-region-specific oligonucleotide primers must be manufactured. This process adds significant cost and time constraints. In spite of all of this, false negative results can arise due to clonal evolution or the appearance of sub-clones not evident at diagnosis, both of which decrease or eliminate the utility of the primers customized to the original leukemic genotype.[17]

Regardless of what target is used, PCR techniques are capable of sensitivities ranging from detection of one leukemic cell in 10^3 to 10^6 normal background cells. Quantitation is difficult with fusion transcripts, but it is quite accurate using gene rearrangements, and the advent of real-time quantitative PCR technologies may expedite and further tailor the method of MRD quantitation. In general, it must also be noted that even PCR-negative patients may harbor between 10^4 and 10^5 leukemic cells considering the increased but still finite sensitivity of the technique, and so a negative PCR result should not be interpreted as the complete absence of leukemic cells. In addition, PCR-positive patients may be identified as leukemia-free if the most stringent cleanliness and procedural standardization has not been followed. In terms of sampling error, not only is marrow aspiration or biopsy limited, but it is reasonable to assume that

MRD levels can fluctuate both above and below the limits of PCR detection, thus leading to a temporal variability that could produce false-negative results.[80] The theoretical question of whether or not there ever can be elimination of all leukemic cells, and whether or not this matters clinically, soon becomes apparent. Is there a finite limit to sensitivity? Can any chemotherapy regimen ever kill every malignant cell? Will it eventually have to in order to meet a future standard of care? Most likely there is a degree of elimination at which remaining leukemic cells either lose their ability to get an "exponential head start" or the host immune system is able to chronically suppress any further clonal expansion.[49]

MRD methods of detection: flow cytometric immunophenotyping

Targets for flow cytometric detection of MRD rely on leukemia-associated immunophenotypes associated with the patient's malignant cells. Essentially, a pattern of surface marker expression unique to the clone and not expressed by normal marrow or blood constituents is sought at the time of diagnosis by flow cytometry of cells derived from the marrow aspirate. This proves relatively straightforward for T-lineage ALL, by virtue of the presence T-cells outside of the thymus with a ubiquitous leukemia-associated immunophenotype in the form of CD3 or CD5 and TdT positivity.[20,76,81] However, isolation of aberrant expression patterns can be more complicated for B-lineage ALL, especially when the malignant cells have been derailed early in the differentiation process. Once a distinguishing immunophenotype has been identified, post-remission marrow samples can be assayed with high sensitivity in order to detect residual leukemic cells expressing the same clonal pattern of surface markers.

As stated above, it can be difficult to distinguish the immunophenotypic characteristics of B-lineage ALL cells from those of normal early lymphoid progenitors, also known as hematogones. Current understanding of leukemogenesis is founded on the principle that leukemia originates from the clonal expansion of a transformed hematopoietic cell that has been arrested at a particular stage of differentiation. The implication of this is that B-lineage leukemic blasts are directly related to their normal counterparts in lymphopoiesis. It has been documented that normal cells with aberrant immunophenotypes, such as co-expression of T-cell or myeloid markers and simultaneous expression of both early and late differentiation antigens, can be found in normal bone marrow at the 1 in 10^4 sensitivity level.[18,82] Just as with morphologic screening, these relatively immature cells can also be immunophenotypically confused with

leukemic blasts during flow cytometric assays. And, the issues is made more complicated by the fact that numbers of these progenitor cells can escalate significantly in regenerating marrow following chemotherapy or bone marrow transplant. Beyond this, hematogones are present in higher proportions in the marrow of young children at baseline, and this population bears the burden of most ALL cases.[83-84]

Some recent work has suggested that leukemic cells can be distinguished from normal B-cell precursors by quantitative expression levels of certain antigens using so-called comparative phenotype mapping.[18,85-86] Other authors have reported methods using non-quantitative expression patterns. One report found increased expression of markers such as CD-58, creatine kinase-B, ninjurin-1, REF1, calpastatin, HDJ-2, and annexin-VI in B-lineage ALL cells when compared to B-cell precursors, and CD-58 staining correlated well with PCR of Igh gene rearrangements.[76] Another report found that simultaneous expression of CD-19, CD-10, and either CD-34 or TdT exhibited some sensitivity. This author suggested that quantitative differences in antigen expression could be used in between 30-50% of cases to distinguish normal and leukemic cells, and that with larger panels of markers this might be raised to roughly 85%.[20] Weir et al.[19] used a four-color flow cytometry apparatus to distinguish up to 99% of hematogones from B-precursor ALL cells in a sample of 82 cases. Another author has suggested that leukemia-associated immunophenotypes (LAIP) can be identified in up to 90% of B-lineage ALL.[87] Regardless of the progress being made in identification, the issue of immunophenotypic overlap between ALL cells and marrow precursors remains influential in daily practice. In recent years it has been estimated that approximately 2/3 of pediatric ALL patients can be monitored for MRD with any prognostic relevance when using standard immunophenotyping techniques.[74,88] Even this generous estimate leaves roughly 30% of ALL patients with a diagnostic dilemma regarding MRD detection. A highly sensitive technique can make for ambiguous results when specificity is lost.

Flow cytometric immunophenotyping is an efficient technique in that it is particularly rapid. It is fundamentally an excellent method for quantification of disease, as its historical roots were based in cell counting. Additionally, it provides a broad overview of hematopoietic status as part of routine processing. One additional feature that distinguishes its utility from that of PCR is the ability to distinguish viable from apoptotic cells within a given sample. Difficulties with flow IP include false-negative results due to immunophenotypic shifts in the leukemic clone, although the confounding effects of this issue can be

reduced by the use of multiple markers, when available. A more significant lineage switch, with ALL relapsing as AML, or a secondary hematological malignancy due to the mutagenic effects of chemotherapy may also occur.[1,20] False-positive results arise mainly through the inappropriate choice of markers to delineate normal from malignant cells due to human error. Finally, there is significant complexity involved in the interpretation of immunophenotype data once it has been gathered and analyzed, which is both time and labor consuming, as well as providing fodder for misinterpretation.[17]

Two general variables influence the effectiveness of MRD detection by flow cytometric methods. The first, as outlined above, is the degree of morphologic or immunophenotypic difference between malignant and normal background cells. The second is the quantity of cells that can be analyzed. Flow allows for a sensitivity of up to 1 in 10^6 cells if a sufficient number of cells are analyzed (the magic number being roughly 10^7 total cells) and if the fluidics system of the cytometer is painstakingly maintained. However, more realistic sensitivity estimates for clinical (non-research) machines hover around 1 in 10^4 to 10^5 cells.[20] In everyday practice, with approximately 10^6 cells to assay, and considering the need for at least 10 to 20 data points for adequate interpretation, a sensitivity of 0.001% (1 in 10^5 cells) should be achievable.[76]

MRD prognostic significance

It has been repeatedly documented in recent years that MRD has prognostic significance for patients with ALL (see below). The presence and quantitative level of residual leukemic cells at multiple time-points predicts likelihood of relapse and event-free survival with great power, and the predictive value remains robust even when adjusting for other prognostic factors. The following paragraphs offer a review of multiple studies conducted regarding the prognostic value of MRD detection using either PCR or flow cytometric technology.

MRD prognostic significance: PCR

PCR-based MRD detection does possess prognostic significance in ALL. VanDongen et al.[9] conducted a prospective study of 240 childhood ALL patients being treated in Europe. Bone marrow samples were collected at up to 9 time intervals both during and after treatment, and PCR of patient-

specific Igh and TCR gene rearrangements, as well as TAL1 deletions when applicable, was conducted. All patients had appropriate targets for PCR, and 62% of patients had either 2 or 3 available targets. Of the 36 patients that relapsed, 94% retained at least one stable PCR target. The loss of targets mainly affected T-lineage patients, likely due to continued clonal rearrangements. Only one B-lineage ALL patient lost viable targets completely. Data showed that positive MRD status during treatment led to a 5 to 10-fold increase in relapse rate. The difference was highlighted the most at detection time-point 5 before the end of treatment, with 9% of MRD-negative patients vs. 86% of MRD-positive patients from this group relapsing at 3 years. For those who remained MRD-positive after treatment was completed, 5 of 6 patients relapsed, although 9 of 148 MRD-negative patients eventually relapsed as well. Upon multivariate analysis, presence of MRD was found to be an independent prognostic factor at each of the first 5 time points used in the study. In addition, a quantitative correlation was found, with distinct degrees of MRD having specific prognostic significance. Each 10-fold increase in degree of MRD produced a 2-fold increase in relative risk of relapse. Importantly, this relationship remained significant between each treatment classification group (standard-, moderate-, and high-risk), and the prognostic impact was consistent even after controlling for age, sex, initial WBC count, and immunophenotype. This work clearly demonstrated the prognostic value of MRD detection in ALL, and made the important step towards association of MRD quantification with outcome variability.

Cave et al.[8] conducted a prospective study of 178 patients with childhood ALL, collecting bone marrow specimens at 4 time points during the first 6 months after remission induction. PCR amplification of patient-specific gene rearrangements was again employed for detection of MRD. At least one target was available in all patients, with 26% possessing more than one probe. Compared to the MRD-negative patients, those with positive MRD status before consolidation therapy had a relative risk of relapse of 4.9. Positive MRD status continuing after consolidation amplified the effect, with the relative risk of relapse jumping to 15.0. Among relapsed patients, those with detectable MRD at the completion of induction therapy displayed a shorter time to relapse than those without MRD. In an attempt to quantify the effect, patients were stratified by MRD level into groups with less than 1 leukemic cell per 10^3 normal marrow cells, between 1 per $10^3$and 10^2, and greater than 1 per 10^2. As expected, the risk of relapse increased sequentially with level of MRD. Not surprisingly, survival was also related to presence and level of MRD;

those with MRD-positive status were burdened with a 10-fold greater risk of death within the studied time frame than those with no detectable MRD. Again, the prognostic significance of MRD remained stable in bivariate analyses with risk-group stratification, age, WBC count, and immunophenotype.

Gruhn et al.[12] conducted a smaller study of 26 children with B-lineage ALL in which post-induction marrow samples were analyzed for MRD. A quantitative semi-nested PCR procedure was utilized to amplify Igh gene rearrangements. After completing PCR studies, MRD-positive patients were divided into a low-level group, with less than 2 malignant cells per 10^5 normal marrow cells, and a high-level group, with greater than 2 per 10^5. According to the collected data, all patients with low or undetectable levels of MRD remained in complete remission with a median follow-up of 5.25 years, while all patients with higher levels subsequently relapsed. In spite of the small sample size, this author concluded that level of MRD following induction therapy predicts outcome in childhood B-lineage ALL. The suggestion was also posited that it may be advisable to utilize this factor for a new form of risk stratification, in which post-induction MRD-positive patients should be given more intensive therapy and possibly even bone marrow transplantation. It is, however, difficult to set consistent cut-off points between low- and high-risk patients, as these demarcations may be influenced by intensity of induction therapy, time of marrow examination, and even method of MRD detection.

Another small study was conducted more recently by Gameiro et al.[10] using a sample of 52 children with ALL (44 of whom had common or pre-B ALL). PCR was attempted utilizing both antigen receptor gene rearrangements and fusion transcripts, when applicable. The patients involved were monitored for a median of 45 months, with marrow examinations within 4 time periods, 0-2 months, 3-5 months, 6-9 months, and 10-24 months. Seventeen patients relapsed, but only 11 were able to be followed. Of this group, 3 had lost their original clonal PCR target, demonstrating the difficulties caused by clonal evolution when using PCR analysis. The remaining 8 patients retained their original PCR target(s) and were able to be followed. During the first 24 months, MRD detection correlated with outcome for all time periods, and proved to be not only independent but also more accurate in predicting outcome than age and WBC count. MRD-positivity was associated with an increased relative risk of relapse, with this impact growing stronger with time. At each time-block the relative risk of relapse for MRD-positive vs. negative patients was 1.89 vs. 0.72, 2.20 vs. 0.82, 2.65 vs. 0.65, and 2.16 vs. 0.70. Clearly, the greatest association

was observed at the 6-9 month time period, corresponding to the start of maintenance therapy. Among those who relapsed, at all 4 time points the number of MRD-positive patients exceeded the number of those with undetectable MRD. As with the aforementioned studies, 3 subgroups of MRD-positive patients were created including those with greater than 1 leukemic cell per 10^2 normal marrow cells, those with between 1 per 10^3 and 1 per 10^4, and those with less than 1 per 10^4. Level of MRD-positivity was significantly associated with disease-free survival at all time periods.

Eckert et al.[11] conducted a retrospective study of 30 children with ALL utilizing PCR amplification of Igh and TCR gene rearrangements. Patients were divided into subgroups with MRD quantified as greater than 1 leukemic cell per 10^3 normal marrow cells or less than 1 per 10^3. At day 36 of therapy, the probability of event-free survival at 6 years was 0.86 for the low-level MRD patients versus 0% for those with higher levels of MRD. These results suggest that the extent of early response to therapy, as evidenced by levels of residual leukemia, can be used to predict long-term outcome in ALL patients. Those whose MRD levels are low at early time points may have a more brisk response to therapy, and may therefore have a better eventual outcome. Again, this information may be helpful for risk stratification and subsequent adjustment of treatment intensity to better match prognosis.

Another smaller retrospective study conducted by Roberts et al.[15] sequentially followed 24 children during their first clinical remission from B-precursor ALL during a 5 year follow-up period. MRD was monitored using quantitative PCR of Igh gene rearrangements. Among the patients who relapsed, levels of residual leukemic cell DNA were significantly higher by quantitative PCR when adjusted for time. However, the mean level of leukemic cell DNA at any specific time was not associated with probability of relapse, suggesting the need to follow time trends. Notably, 15 of 17 patients who remained in CCR and all 5 relapsed patients had some degree of detectable residual disease. However, the PCR results did show a temporal pattern of increasing MRD levels in those who relapsed. Unfortunately, no threshold level of residual leukemic DNA was significantly associated with relapse. However, autoregression calculations demonstrated significant association between a trend of increasing levels of MRD and eventual re-emergence of disease. Thus, a predictive value before relapse was found. The fact that so many patients in CCR remained MRD-positive at some level further challenges the dogma of equating cure with complete

elimination of leukemic cells. With higher sensitivity methods comes the detection of more minute levels of disease that may, at some threshold, lose clinical significance.

Goulden et al.[16] studied 66 children with ALL retrospectively, but added the additional control of limiting the study set to those with only standard-risk ALL. Again, PCR amplification of Igh and TCR gene-rearrangements was performed. Bone marrow aspirates were collected at 1, 3, and 5 months into treatment, and the presence or absence of MRD was documented for both those in CCR and those who relapsed. At these sequential time intervals for patients in CCR vs. those who relapsed, MRD-positivity was present in 32% vs. 82%, 10% vs. 60%, and 0% vs. 41% respectively. It is clear that MRD was more prevalent among those who relapsed, and that a downward trend with time led to eventual MRD-negativity for those in CCR. In these standard-risk patients without additional adverse prognostic factors MRD-positivity was still significantly associated with relapse.

A retrospective analysis of 90 ALL patients, 19% of whom possessed the TEL/AML1 translocation, was performed by DeHaas et al.[89] The aim was to determine whether or not the presence of MRD retained its prognostic value even with the presence of a beneficial prognostic factor such as the TEL/AML1 rearrangement. PCR was performed using antigen receptor gene rearrangements as per usual, and levels of MRD were measured after induction therapy. Patients who went on to relapse had significantly higher levels of MRD when compared with those who achieved CCR, and indeed this same pattern held true for the patients with the TEL/AML1 translocation. This study serves as one example of the persistence of MRD as a prognostic factor despite the presence of other prognostic variables.

MRD prognostic significance: flow cytometric immunophenotyping

The prognostic value of flow cytometric detection of MRD has also been demonstrated in the literature. Dworzak et al.[14] conducted a prospective study of 108 children with ALL using flow cytometric assessment of bone marrow samples at 4 time periods during the first 6 months of treatment. MRD was quantitatively assessed based on both the number of blasts relative to normal nucleated marrow cells and by absolute measures of leukemic cells per microliter. At all time points except day 15, the presence of MRD was associated with greater likelihood of relapse. In fact, 99% of assays using day 33 marrow had sufficient sensitivity for outcome prediction. Importantly, incidence of relapse was found to correlate with

distinct levels of MRD-positivity. The author found a high predictive value in combining MRD data from day 33 and from week 12. The earlier data provided a sensitive pre-definition of high-risk patients, while the later data allowed for estimation of the kinetics of MRD evolution between two time points. Overall, MRD measures by flow cytometry proved to be an independent, and even overriding, prognostic factor in multivariate analyses using other risk factors, including the Berlin-Frankfurt-Munster (BFM) risk stratification scheme. Based on these results, the author suggests a new multilayered risk stratification system, placing patients at high conventional risk (HR) into an MRD-positive group, with particularly bad outcome and the need for alternative therapies, and an MRD-negative group with a relatively good prognosis using intensive chemotherapy. A second set of patients would be those with low conventional risk (SR or MR), who could be divided into an MRD-positive group, with an increased chance of relapse and the need for therapy intensification, and an MRD-negative group who would have an excellent outcome with standard chemotherapy protocols. The author raises an interesting point in that the predictive value of MRD detection may vary with the intensity and schedule of chemotherapy. This type of variable warrants further investigation. Also of interest, this study substantiated the previously documented difficulties reported by many authors regarding the inability to distinguish lymphoid precursors from B-lineage leukemic blasts by immunophenotyping.

Another prospective study was conducted by Coustan-Smith et al.[6] involving 195 childhood ALL patients in newly diagnosed remission. Bone marrow samples were collected at the end of remission induction and at 3 later time points and then subjected to immunophenotypic analysis by flow cytometry. At all measured points during clinical remission, the presence of detectable MRD was associated with a higher rate of subsequent relapse. In particular, patients with high levels of MRD at the end of induction or at week 14 of continuation therapy had an especially poor outcome. Among patients who were MRD-positive at the end of induction, those who remained MRD-positive through week 14 of continuation therapy relapsed at a rate of 68%, whereas those who became MRD-negative by week 14 of continuation had a relapse rate of only 7%. As noted in other studies, the predictive strength of MRD detection remained significant even after adjustment for other prognostic factors. Some presenting features, such as the patient's age and presence of certain genetic anomalies, are related to the rate and extent of initial cytoreduction, but notably, MRD detection at the end of induction therapy continued to identify patients at

higher risk for relapse regardless of rapid clearance of leukemic blasts. In spite of fairly compelling results, this author suggested the use of tandem flow cytometry and PCR analysis in order to eliminate the risk of false-negative findings due to immunophenotypic shifts or clonal evolution.[6,90] Overall, however, the results clearly demonstrate prognostic utility for the flow-based measurement of MRD.

Another study by the same author involved prospective analysis of 158 children with ALL using flow cytometry to identify and follow leukemia-associated immunophenotypes.[74] Marrow samples were collected at the end of induction, as well as at weeks 14, 32, and 56 of continuation, and at week 120 (the end of therapy). At each time point analyzed, detection of MRD was significantly associated with a greater likelihood of treatment failure and disease relapse. The predictive value of MRD remained robust after adjustment for age, WBC count, and presence of the Philadelphia chromosome or MLL gene rearrangement. The overall conclusion was that immunological detection of MRD by flow cytometry at any point during the course of treatment is a powerful predictor of relapse in children with ALL.

Just prior to Coustan-Smith's 1998 report, Farahat et al.[75] published a paper detailing a retrospective study of 53 children with B-lineage ALL. Patients were monitored for MRD with the use of flow cytometry to detect CD10+, CD19+, TdT+ cells in the marrow at several points during therapy. MRD was detected despite negative morphology in many cases, leading the authors to conclude that quantitative flow cytometry was a superior technique for assessment of remission status. There was a statistically significant difference in disease-free survival rates between MRD-positive and MRD-negative patients at the start of morphological remission. Presence of MRD reliably predicted early relapse, and thus a correlation between MRD status and outcome was confirmed.

Borowitz et al.[13] attempted to correlate MRD with other risk factors in 1016 children with precursor-B-cell ALL. The authors utilized 4-color flow cytometry to analyze marrow samples at the end of induction therapy, while simultaneously screening for other risk factors in ALL such as clinical risk stratification, cytokinetic responsiveness, and genetic abnormalities within the leukemic clone. MRD levels correlated with NCI risk-group stratification, showing that NCI high-risk patients were more likely to be MRD-positive than the standard-risk group. This suggests that poor clearance of leukemic cells during early therapy may partially explain traditional high-risk characteristics. Along these same lines, patients demonstrating a slow early response to chemotherapy as judged by day 8 marrow morphology were more

likely to be MRD-positive at the end of induction. Interestingly, flow cytometry of day 8 blood samples and morphologic examination of day 8 marrow proved to be independent predictors of later MRD-positivity. The authors suggest that monitoring of day 8 blood by flow methods may identify which patients might benefit from marrow examination, thus saving some patients from unnecessary aspirations and ensuring adequate surveillance for those at higher risk. Genetic factors were also assessed in this report. It was found that Philadelphia chromosome-positive patients tended to have higher levels of MRD. Those with the favorable TEL/AML1 translocation were found to have relatively low rates of MRD-positivity, consistent with other reports that patients with this anomaly tend to be rapid responders to conventional chemotherapy.[91] Interestingly, patients with the favorable trisomies 4 and 10 were found to have a 2- to 3-fold *higher* incidence of MRD-positivity when compared with standard ALL patients. Further work must be done to determine if MRD-positive trisomy-positive children have higher relapse rates. However, with the typically beneficial nature of trisomies 4 and 10, early intensification of treatment for MRD-positive patients would likely be overzealous. This curious development lends caution to assumptions that MRD status may trump all other prognostic factors.

In an attempt to confirm the significance of MRD detection among other age groups, Vidriales et al.[92] studied 102 adolescent (age greater than 14) and adult patients with ALL using flow cytometric immunophenotyping of day 35 marrow samples. Multivariate analysis showed that immunologic evaluation of day 35 marrows was actually the most robust independent prognostic factor for these patients. The authors concluded that, in combination with age, WBC count at diagnosis, and presence of genetic anomalies, MRD detection allowed for very informative prediction of relapse-free survival. This serves as evidence that flow cytometric MRD studies have broad applicability to ALL patients, regardless of age-group.

MRD and DNA ploidy

It has been shown that results and sensitivities of MRD detection correlate well between PCR and flow cytometric immunophenotyping.[90] There are advantages and disadvantages to both, and in time perhaps all samples will be subjected to both modalities, as has been suggested by some.[6,90] However, many institutions, including our own, rely on flow cytometry technology for MRD detection. In spite of

the success in measurement of MRD by flow cytometric methods and its clear prognostic value, the previously mentioned difficulties due to proliferation of B-cell precursors in therapeutic bone marrow remain a challenge in a substantial number of ALL cases. In order to clarify this area of blurred specificity, particularly in the realm of B-lineage ALL, it is necessary to find additional distinguishing characteristics of residual leukemic cells that do not rely on overlapping immunophenotype. With up to 40% of B-lineage ALL cases demonstrating aneuploidy, the presence of abnormal DNA content may be a variable that can be used in at least a proportion of cases to assist in the accurate detection of MRD. DNA ploidy analysis can be conducted using a flow cytometer, and has been used for quite some time to characterize both solid and hematological tumors. The tandem use of flow cytometric immunophenotyping and DNA ploidy analysis in B-lineage ALL is a logical combination, as both assays can be conducted rapidly with the same apparatus, and both sets of data can be interpreted together.

History of flow cytometry

Since the 1930's, when the evolution of flow cytometry began, the technique has been developed and refined to allow researchers and clinicians to analyze great numbers of cells rapidly and accurately.[93-94] With the invention of the microscope in the 1600's, the development of better tissue stains in the late 1800's, and eventually the discovery of fluorescent markers and photodetectors to measure their output, cytometry has developed, first from direct observation of cells, to rapid analysis using computer technology. The first steps towards a "flowing" system, in which cells are analyzed as they move in a fluid stream, began in 1930's with Andrew Moldavan's invention of a device to count erythrocytes and Torbjorn Caspersson's progressive use of microspectrophotometers to measure the UV absorption of cell nuclei.[93,95] This gave way in the 1950's to the familiar Coulter technology that has allowed rapid counting of cells in a liquid stream based on impedance characteristics.

In the 1960's this technology was adapted by Louis Kamentsky with the addition of a microscope-based spectrophotometer which was calibrated to measure UV absorption and the scatter of blue light from cell flowing past an objective. Further along in the decade, Dittrich & Gohde furthered the process by creating a device that could measure ethidium bromide-stained nuclear DNA fluorescence and create intensity-derived histograms of DNA content. This procedure serves as a foundation for the current DNA

ploidy analysis that has been mentioned above. From here, the goal shifted to cell sorting, and in the late 1960's newly emerging ink-jet technology was harnessed in conjunction with Coulter technology by Mack Fulwyler at the Los Alamos Laboratories to create an instrument capable of sorting erythrocytes. All of these developments led to creation of the first fluorescence detection cytometer in 1969 by Marvin Van Dilla, also at Los Alamos Laboratories. By 1970, separation of leukocytes began to be achieved, and from there the technique grew.[95] With time, the analytical speed of current machines has exceeded that of the early slide-based microspectrophotometers by 4 to 5 orders of magnitude.[96]

Development of fluorochromes and monoclonal antibody technology has led to the use of flow cytometry for research and clinical purposes, but perhaps its most powerful use has been in the field of hematopathology. Here three-color, and now four-color, flow cytometry using fluorochromes attached to a wide variety of monoclonal antibodies against leukocyte CD markers has allowed for precise identification of leukemic cells and delineation of maturational status.

Flow cytometry principles

Flow cytometry has applications in both solid and hematological tumors. In either case, the utility of the technique lies in rapid analysis of a large number of cells flowing single-file in a fluid stream exposed to high-intensity laser light.[94]

The basis for immunophenotyping in ALL traces back to the mid 1970's, when flow technology was used at the ICRF Tumor Immunological Unit in London to test an antiserum which reacted with a leukocyte surface antigen termed CALLA (for common ALL antigen), now known as CD-10.[97] Evolution of monoclonal antibody technology and a series of leukocyte-typing workshops led to delineation of an extensive list of CD markers. Multiparameter flow cytometry was then poised to trace the steps of maturation among the lymphoid cell lineage. In this way, normal B-lymphoid development was worked out, and the highly controlled sequential acquisition of leukocyte surface markers was outlined. At the same time, it became clear that while normal differentiating cells express surface antigens in an orderly and predictable fashion, leukemic cells follow a dysregulated and confused pathway.[97] From here, the clinical use of flow immunophenotyping became clear, as it promised a more objective criterion to support the morphological diagnosis and classification of ALL.[98] B-lineage precursors commit to their pathway with

the expression of surface CD10, CD19, and CD22, nuclear TdT, and cytoplasmic CD79a. As they progress, these early B-cells sequentially begin to lose CD34 and nuclear TdT, express decreasing amounts of CD10, and gain reactivity for CD20. Later, cytoplasmic immunoglobulin mu heavy chains are produced, until eventually light chains are made and complete secretory and surface immunoglobulin molecules can be identified. Aberrancy during this process, either through cross-lineage expression patterns or asynchronous antigen expression, can identify neoplastic cells.[98] Analysis of extracellular targets, such as leukocyte surface antigens, involves incubation of blood or marrow with fluorochrome-labeled monoclonal antibodies to specific surface markers. By this process, the presence or absence of markers can be determined based on positive or negative fluorescent staining. Cells exposed to antibody are passed single-file through a focused light source, typically an argon laser. Interaction of fluorochrome molecules with laser light allows for excitation of electrons. After excitation, the subsequent orbital energy changes associated with electron transit back to resting state produce emitted light, and the cells themselves produce scatter of the laser photons. At this point, light is separated by wavelength using a series of mirrors and filters and directed towards fluorescence photodetectors, which allow for a quantitative measure of signal that can be inputted into scatter plots and histograms for description of the cellular population.[94] This technique is quite powerful in describing the immunophenotype of leukocytes, and is highly applicable to the diagnosis and classification of ALL.

By the mid 1950's, before immunophenotyping was a reality, it was already clear that malignant cells were likely to have greater amounts of nucleic acid than normal cells, and this realization has continued to be demonstrated in many different tumor types.[93,99-104] Aneuploidy was a common feature of many tumors, solid and hematological, and preliminary studies began to show that aneuploid cell lines and those with high S-phase fractions were predictive of poor outcome in a wide variety of tumors.[96] The pattern of karyotypic instability that leads to spontaneous progression from normal euploid to aneuploid to neoplastic began to be elucidated.[105] Intracellular flow cytometric studies can be applied to assay cellular DNA content, as well as some cytoplasmic markers. To conduct these assays, the cell membrane is first permeabilized using various detergents, alcohols, or paraformaldehyde. Subsequently, fluorochromes are introduced that stain the desired target. In the case of DNA, propidium iodide or eithidium bromide are commonly used as fluorescent stains after removal of RNA. As above, the cells are passed single-file

through the laser light source and levels of fluorescence are detected and quantified.[94] This technique is useful in ALL as a method of determining DNA ploidy.

The cell cycle consists of several phases including Gap0 (G0), Gap1 (G1), Synthesis (S), Gap2 (G2), and Mitosis (M). The DNA content of cells is dependent on what phase of the cell cycle they are in. Cells in G0, G1, and M phase are diploid (2n), whereas cells in G2 and at the end of S phase have a higher DNA content (4n).[106] As mentioned before, neoplastic cells, including those of ALL, may have abnormal DNA content due to gain or loss of sets or fragments of chromosomes. This is known as aneuploidy, and it can be detected through the flow cytometric methods described above. DNA histograms can be derived from cytometric measures of DNA fluorescence in order to isolate the proportion of cells in different stages of the cell cycle and to quantify populations with abnormal DNA content. Using the data gathered from the cytometer, the percentage of aneuploid cells, the rate of proliferation of neoplastic cells (as represented by the proportion of cells in S, G2, and M phases), and the DNA index (the ratio of the DNA content of neoplastic cells to that of normal cells in the sample) can be described.[94]

The use of flow cytometry for DNA index calculation lends great sensitivity and speed to this process. The technique is used widely for prognostic purposes once the diagnosis of ALL has been made, and it is simple and reliable. Along with the fact that a significant percentage of ALL cases exhibit aneuploid cell populations, these positive attributes make the use of DNA ploidy analysis an attractive consideration as a means to detect minimal residual disease. In the 20 to 40% of patients with aneuploidy, the use of an "immunophenotype-neutral" study, such as ploidy, may provide important additional information in MRD detection, especially if the case involves a B-lineage ALL without a unique phenotype. Because ploidy studies do not rely on markers that overlap between normal immature lymphoid precursors and leukemic blasts, they could potentially add specificity to flow cytometric immunophenotyping. This possibility forms the foundation of the question addressed in this report. The following is a retrospective analysis of all cases of ALL seen at our institution between 1991 and 2003. Flow cytometric immunophenotyping and DNA ploidy analysis were conducted on all patients initially, and in follow-up during treatment. We attempted to answer the question of whether flow cytometric DNA ploidy analysis is a useful adjunct in the detection of MRD for patients with B-lineage ALL.

PURPOSE

The purpose of this research project is to determine the utility, or lack thereof, of flow cytometric DNA ploidy analysis as a means of detecting minimal residual disease or early relapse among patients with treated B-lineage acute lymphoblastic leukemia.

METHODS

Patients

Data regarding the flow cytometric analysis of peripheral blood and bone marrow from all patients diagnosed with ALL between 1991 and 2003 at Yale New Haven Hospital were retrieved from our hematopathology database using the *Access* application (Microsoft, Redmond, WA). Cases were identified using the query function and the search terms "ALL" and "Acute Lymphoblastic Leukemia". This retrieval identified 114 pediatric and adult patients diagnosed with or followed-up for ALL at our institution during the specified time period. As a part of standard diagnostic procedure, flow cytometry had been utilized to determine the presence, immunophenotype, and DNA ploidy of neoplastic cells in samples from all patients identified. Results of DNA ploidy analysis and immunophenotyping of samples from days 14 to 28 and from post-remission studies were available through the database. Additional follow-up data regarding the occurrence of post-therapy or post-remission disease were obtained from patient files kept in the hematopathology department. The 114 patients were divided into four groups designated by immunophenotype and ploidy status: *euploid with normal immunophenotype, euploid with aberrant immunophenotype, aneuploid with normal immunophenotype, and aneuploid with aberrant immunophenotype* (table 1).

Aneuploidy was defined as a DNA index greater or less than 1.0. "Normal" immunophenotypes were defined by expression of markers characteristic of precursor B-cells, regardless of signal intensity. This set of markers included CD10, CD19, CD45, CD34, and CD20. Patients were included in this group regardless of whether their leukemic clone normally expressed, overexpressed, or underexpressed these markers. Some authors have labeled ALL immunophenotypes with different quantitative expression patterns as aberrant since these variations are increasingly able to be identified using four-color flow cytometry[18]. We chose to include these ALL cases in the normal category in order to compare them with those expressing myeloid or T-lineage markers, which can be more reliably detected by flow cytometric methods. Due to the nature of the study hypothesis, we focused our attention on patients with documented aneuploid cell populations.

Immunophenotyping

Peripheral blood and bone marrow specimens collected in ethylenediaminetetraacetic acid (EDTA) were analyzed using a FACScan cytometer (Becton Dickinson, San Jose, CA). Either three- or four-color immunofluorescence studies were performed using monoclonal antibodies to leukocyte CD markers conjugated to fluorescein isothionate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), Cy5.5, and allophycocyanin (APC). "Calibrate beads" in conjunction with FACSComp software (Becton Dickinson) were used to ensure adequate optical alignment and compensation within the flow cytometer. Isotype-matched negative controls conjugated to each fluorochrome were also employed in order to ensure proper function of reagents and absence of non-specific signal production. Initial diagnosis of ALL was conducted using a standard diagnostic panel of monoclonal antibodies. Although alterations in marker selection evolved over the period of time that the data were collected, the general approach included antibodies against CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD13, CD14, CD16, CD19, CD20, CD33, CD34, CD41a, CD45, CD56, CD64, CD79a(c), CD117, myeloperoxidase, TdT, glycophorin, HLADr, and kappa and lambda light chains. Further distinction was provided with antibodies against cytoMu for those with pre-B ALL, and with anti-IgM, IgD, IgG, FMC7, CD23, CD11c, and CD43 for mature B-cell disease. Follow-up studies utilized custom panels specific to the initial leukemic immunophenotype.

DNA Ploidy Analysis

Cells isolated on Ficoll-Hypaque were fixed in ice-cold 70% methanol and exposed to 100μg/ml of RNase in order to degrade any RNA present within the sample. The cells were then stained with a saturating concentration (40μg/ml) of propidium iodide in order to fluorescence-label the sample DNA. Two aliquots of each sample were stained, and the second sample was mixed in a 1:1 ratio with normal human mononuclear cells that were processed identically and served as an internal standard. After staining, the samples were protected from light and analyzed within two hours. Flow cytometric ploidy analysis was performed with a FACScan flow cytometer using CellFIT software (Becton Dickinson). For each sample, $1.5x10^4$ cells were analyzed. Single cells were distinguished from cell clumps by a doublet discrimination model utilizing pulse-area vs. pulse-width measures. The resulting DNA histograms were evaluated using

the SFIT mode. DNA index was calculated by dividing the modal fluorescence channel of the G_0/G_1 peak of abnormal cells by the modal fluorescence channel of the residual G_0/G_1 normal cells present in the sample.[107]

Mixing studies

Cells from the human monocyte cell line U937 with a known mean DNA ploidy of 1.46 were obtained. These cells were then mixed in specific proportions with peripheral blood samples from patients with no hematologic abnormality and normal DNA ploidy. Six mixing proportions were utilized, including 20.00%, 4.00%, 0.40%, 0.04%, 0.004%, and 0% U937 cells. These mixed samples with a known percentage of aneuploid cells were then subjected to DNA ploidy analysis as detailed above, such that a comparison could be made between the known and analytically determined percentage of aneuploid cells in each sample with a DNA index of 1.46. The standard cell cycle modeling program associated with our DNA ploidy analysis software was also utilized in order to determine if this would provide more accuracy in ploidy measures due to control for cell cycle variation between the normal and aneuploid cell populations.

RESULTS

DNA Ploidy Analysis

One-hundred and fourteen patients were diagnosed or followed-up for ALL at our institution from 1991 to 2003. An aneuploid population of cells was detected in the marrow or blood samples of 37 (32%) of these patients. Within this group, samples from 24 patients revealed leukemic clones with normal immunophenotypes, and samples from 13 patients revealed a clonal population with aberrant immunophenotype (table 1). Marrow specimens were monitored between days 14 to 28 and post-remission in most patients.

The detection of MRD at these stages in patients with normal immunophenotypes is summarized in table 2. Patients 1-5 in table 2 experienced post-remission relapse. It is notable that DNA ploidy analysis revealed regenerated aneuploid populations of cells in all patients who relapsed. In fact, in all cases the percentage of non-erythroid marrow cells identified as aneuploid was higher than the percentage of cells identified as matching the original leukemic immunophenotype. Patients 1-3 were found to have less than 10% of cells expressing the original leukemic immunophenotype by flow cytometry at post-remission follow-up. Because these individuals presented with normal (i.e. non-unique) ALL immunophenotypes, these percentages are estimates. Due to similarity with normal B-cell precursors in post-therapeutic bone marrow, a definitive diagnosis of relapse could not be made by flow cytometric immunophenotyping alone without the presence of unusually high numbers of malignant cells. However, DNA ploidy analysis was effective in revealing the relapse in each of these three patients via the identification of significant percentages of aneuploid cells.

Residual disease was detected in patients 6-8 at days 14 to 28. In this series, DNA ploidy analysis again showed higher percentages of aneuploid cells than flow cytometric percentages of presumably malignant cells. An exception was found in patient 7, where immunophenotyping revealed a slightly higher percentage of cells than did DNA ploidy analysis. However, in all cases where immunophenotyping revealed MRD, ploidy studies did so as well. In fact, in patient 6, immunophenotyping reported less than 5% leukemic cells, consistent with remission, while DNA ploidy

analysis demonstrated 8% aneuploid cells. In this case MRD could not be diagnosed with confidence by immunophenotyping, but was suggested by ploidy analysis.

The presence of aneuploid DNA was useful in distinguishing neoplastic cells with normal immunophenotype in all patients with relapsed or residual disease. In certain cases, notably patients 1,2,3, and 6, the presence of a significant percentage of neoplastic cells could not be identified with confidence through immunophenotyping, largely due to the breakdown in specificity of the assay in the presence of B-cell precursors when the leukemic clone did not possess a unique phenotype.

The detection of MRD at the aforementioned stages in patients with aberrant immunophenotypes is summarized in table 3. As the ALL cell phenotype was sufficiently unique in these patients, differentiation of residual and relapsed disease from "recovering" or normal precursor B-cells could be made with greater confidence compared to normal ALL immunophenotypes. Patients 1-6 experienced post-remission relapse, and in all cases DNA ploidy analysis detected a higher percentage of aneuploid cells than the percentage of cells detected with the original leukemic immunophenotype. In fact, in patients 1 and 2 flow cytometric immunophenotyping detected no leukemic cells in the marrow and blood respectively both at day 14 and post-remission, while DNA ploidy analysis detected a small but distinct population of aneuploid cells in each case. These MRD and relapse events could only be predicted by DNA ploidy analysis and not by immunophenotyping.

In patient 7 MRD was detected at day 14 by both immunophenotyping and DNA ploidy analysis. Again, the ploidy study demonstrated a higher percentage of aneuploid cells than immunophenotyping did of the original leukemic phenotype. This patient eventually went on to complete remission.

DNA ploidy studies never failed to detect MRD or post-remission relapse when it was found by flow cytometric immunophenotyping, whether or not the leukemic cells expressed aberrant markers. In all cases but one, ploidy analysis revealed a higher percentage of abnormal, and likely neoplastic, cells than did immunophenotyping. Additionally, in several cases ploidy analysis was sensitive enough to reveal residual disease or relapse when immunophenotyping failed to do so.

Mixing Studies

Mixing experiments were performed for the purpose of quantifying the sensitivity of the DNA ploidy analysis technique used in the study (table 4). At the highest percentage of aneuploid cell mixing, DNA ploidy analysis was within 0.5% of the true mixing proportion. As the percentage of aneuploid cells was reduced in the mixture, the results were variable, progressing from 1.28% to 0.18% to 0.03% to 0.65% to 0.04% difference between known and analytically determined aneuploid cell percentages. Modeling software, intended to correct for cell cycle variations that may lead to false-positive identification of aneuploidy, was utilized in tandem with the standard mixing study calculations. At the two highest mixing proportions, the software allowed for 0.33% and 0.87% difference between known and analytically determined aneuploid cell percentages. However, the program failed after addition of less than 1% U937 cells to the sample, indicating a potential lack of utility when the population of aneuploid cells is small. Based on these data, it would appear that DNA ploidy analysis using current techniques should achieve a sensitivity of 0.5% at best, with a value of 1% seeming more realistic in routine clinical use.

DISCUSSION

The prognostic importance of MRD detection in ALL is quite clear. Presence or absence and quantity of residual leukemia affects outcome and relapse incidence significantly, regardless of other risk factors. Flow cytometric immunophenotyping is a rapid, sensitive and accurate method of assessing MRD status, but it suffers from an area of decreased specificity in the realm of B-lineage ALL cases. Similarity between leukemic blasts and normal lymphoid precursors in the regenerating bone marrow can cloud immunophenotypic distinction of normal from malignant cells. In light of this, our study sought to investigate whether follow-up DNA ploidy analysis could detect MRD or relapse in patients with B-lineage ALL who had aneuploid cell populations identified at diagnosis. We anticipated that this technique would be especially useful for following patients with non-aberrant immunophenotypes that were similar to normal precursor B-cells.

Our retrospective analysis of cases of B-lineage ALL at Yale New Haven Hospital over the last 12 years revealed 114 patients, 37 (32%) of whom displayed aneuploid cell populations by non-unity DNA index at diagnosis. We were able to identify post-remission relapse or MRD in 15 patients from this group, 7 with aberrant and 8 with normal immunophenotypes.

For those with normal immunophenotype, an aneuploid cell population was always detected when immunophenotyping identified residual disease or relapse. The congruence of results between these two methods lends confidence to the idea that they are both measuring the same population of malignant cells. It would be expected that a regenerating malignant clone originally identified as aneuploid would return with the same altered genetic status. In addition, this congruence suggests that ploidy analysis has at least the same degree of sensitivity as immunophenotyping. In fact, in all but one case, the percentage of malignant cells detected by ploidy exceeded the percentage detected by immunophenotyping, suggesting that ploidy analysis may possess greater sensitivity that immunophenotyping. In this group of patients, ploidy identified on average >8% more detectable malignant cells than did immunophenotyping, with the maximum difference reaching 18%. This increased level of detection led to three cases in which ploidy was able to make the diagnosis of relapse and one case in which it was able to detect MRD while immunophenotyping could not do so with sufficient confidence. These cases serve as examples of the

difficulty created by B-cell precursors in regenerating bone marrow. While some degree of a possibly neoplastic cell population was detectable by immunophenotyping, the extent of the potential clone could only be estimated, due to the fact that malignant cells could not be well-distinguished from normal immature lymphoid cells. Ploidy, on the other hand, detects malignant cells based on a relatively simple clonal characteristic that should not be exhibited by normal marrow constituents. The addition of ploidy analysis allowed for detection of recurrent disease that could have been dismissed using only immunophenotyping in 3 of 5 relapse cases in this group. The same can be said of 1 of 3 cases of MRD. This is a significant improvement in the sensitive and specific detection of MRD and relapse that could potentially be harnessed to provide patients with appropriate early intervention.

For ALL cases with an aberrant immunophenotype, which attenuates the difficulties associated with confusion of lymphoid precursors with blasts, ploidy analysis continued to be a useful tool. Again, detection was congruent between ploidy and immunophenotyping in all cases, suggesting that each technique is measuring the same population of malignant cells. In this group, ploidy detected higher percentages of malignant cells than immunophenotyping in every case. On average, ploidy detected almost 10% more neoplastic cells than immunophenotyping, and in one case ploidy detected 33% more abnormal cells, suggesting a potentially greater sensitivity. In two cases, ploidy analysis detected small but identifiable proportions of malignant cells at both day 14 and post-remission when immunophenotyping detected no abnormal cells at all. These data demonstrate a potential utility for DNA ploidy studies even in B-lineage ALL cases with aberrant immunophenotype.

This is the first investigation of whether DNA ploidy analysis is a useful tool in the detection of MRD in ALL patients. The use of ploidy appears, based on our results, to lend some degree of sensitivity and specificity to MRD and relapse monitoring for individuals with ALL. We have demonstrated several circumstances in which confident diagnosis of MRD or relapse could not be made by immunophenotyping, but where identification of an aneuploid cell population served to confirm the presence of malignant cells. Beyond this, initial results would suggest that ploidy may be a more sensitive means of detecting residual or regenerated leukemic cells. It should be noted that 5 of the 15 patients had immunophenotyping and ploidy analysis performed on peripheral blood rather than bone marrow. In terms of comparing detection

methods, the pattern of results obtained with these samples mirrored those obtained from marrow samples. This suggests that ploidy studies are applicable to peripheral as well as intramedullary sampling.

Mixing studies were carried out using the U937 human monocyte cell line and normal peripheral blood samples in an attempt to better define the sensitivity of DNA ploidy analysis. The results demonstrate that, in spite of an apparently greater sensitivity suggested by increased quantitative detection of aneuploid cells as described above, DNA ploidy analysis may in fact be intrinsically less sensitive than immunophenotyping. Based on the data from this study, a sensitivity of 0.5% to 1.0% may be expected with ploidy analysis, relative to the well documented standard of 0.1% to 0.5% provided by immunophenotypic studies. The principal limitation to sensitivity in ploidy analysis is that some degree of aneuploidy is always present in normal blood or marrow. At any given time, a certain number of cells will be in S-phase, and will consequently exhibit a variable DNA index between 1.0 and 2.0, which would mimic an aneuploid leukemic clone. The final mixing experiment in table 4 demonstrates this, showing that cells in the peripheral blood with a DNA index of 1.46 are present at a level of 0.04%. This effect may be amplified in the marrow, where hematopoeisis is prominent. Cell cycle influences may be attenuated to some extent by using commercially available modeling programs. However, this technology was largely unsuccessful in eliminating the discrepancy in our mixing studies, especially when the percentage of aneuploid cells was low. One additional caveat is that the sensitivity of ploidy analysis is partly dependent on the coefficient of variation (CV) of the aneuploid peak on the DNA histogram. The CV is tighter for leukemic clones than it is for aneuploid cell lines, such as the one we used. Thus, the sensitivities predicted by the mixing data may be slightly better in actual patient samples where leukemic cells are present, but this effect would likely be small. Therefore, at this point in time, there is a boundary to sensitivity in DNA ploidy analysis. While such a limitation does appear to prevent the technology from surpassing the sensitivity of immunophenotyping, it does not eliminate the utility of the technique. As describe above, ploidy analysis continues to identify significant aneuploid cell populations when immunophenotyping cannot be entirely relied upon. In addition, the significantly greater percentage of aneuploid cell detection by ploidy analysis is not necessarily explained entirely by the presence of S-phase cell populations. What is clear, however, is that the higher percentages of aneuploid cells detected by ploidy studies likely do not indicate that the technique has a higher sensitivity.

The most obvious weakness in this study is its small sample size. In spite of retrieving 12 years of data, only 114 ALL patients were identified, with 37 patients exhibiting aneuploid cell populations and 15 of these showing MRD or relapse. Clearly, it is difficult to draw broad conclusions from such a small amount of presented data. However, the congruity and uniformity of results across immunophenotype, age, and sampling location does lend credence to ploidy analysis as a useful technique in MRD detection. Another drawback is the retrospective study design, although gaining sufficient sample size prospectively would have been a difficult proposition. Additionally, only two monitoring periods were included for analysis. Most reports describing MRD prognosis utilize at least 5 time-points, either during or after chemotherapy, for analysis. More data points could have been useful in this study in order to identify clearer trends in consistency between ploidy and immunophenotyping and to document other examples of the success or failure of ploidy analysis to detect disease more sensitively. It would also be beneficial, with sufficient sample size, to separate patients being monitored from peripheral versus marrow sites into separate groups in order to further standardize comparison and prevent confounding in either direction. Some work has shown that MRD detection of B-lineage ALL by immunophenotyping using peripheral blood is not concordant with marrow sampling. Not surprisingly, positive marrow samples are not always accompanied by a positive blood sample, and blood-positivity is associated with a very high risk of disease recurrence.[108]

Many of these weaknesses could be remedied through further investigation using larger sample sizes that not only will provide statistical power, but can be subdivided further and subjected to multivariate analyses. Preferably, such studies would be conducted prospectively along with treatment protocols and would involve sample assessment at multiple time intervals. In light of the widespread use of PCR for MRD detection, it may also be of interest to compare the sensitivity of DNA ploidy analysis with that of PCR amplification. It is likely that such a study could only be completed by one of the larger cooperative cancer groups, such as the Children's Oncology Group (COG).

Beyond difficulties with the study design, DNA ploidy analysis itself has limitations for the purposes of MRD detection. First and foremost, this technique is only applicable to patients with detectable aneuploid cell populations at diagnosis. While all patients may be investigated for the presence of aneuploidy as a matter of initial prognostication, only about 30% of patients will have identifiable

aneuploid populations that can be utilized for follow-up. Additionally, it is not entirely understood whether other events besides re-emergence of a leukemic clone can lead to detection of an increased marrow or peripheral blood DNA index. If other stimuli to aneuploidy exist, it will be necessary to determine whether or not they could confound the use of ploidy analysis in the current context. There is some allegiance among clinicians to the use of either flow cytometric immunophenotyping or PCR for MRD detection, and even without preference, some institutions have access to only one method. This again could be a hindrance to the wide applicability of ploidy analysis, as it will be limited to those settings where a flow cytometer is available and scrupulously maintained.

In spite of its possible limitations, DNA ploidy analysis appears to be a useful technique for detection of relapse or MRD among many patients with B-lineage ALL. Although our sample is small, the presented data indicate that ploidy analysis is capable of detecting residual or relapsed leukemia consistently when compared with immunophenotyping. Ploidy appeared to be a useful, and possibly superior, indicator of relapse or MRD whether or not the leukemic clone exhibited an aberrant immunophenotype, thus exceeding the expectations of our study question. Hopefully these results will be repeated in larger trials, and future patients will be able to benefit from an additional technique to detect unwanted leukemic cells and facilitate effective therapy.

Table 1. ALL patients (n=114) grouped according to immunophenotype (normal vs aberrant), DNA ploidy, and post-remission relapse

Number of patients	Euploid with normal immuno-phenotype	Euploid with aberrant immunophenotype	Aneuploid with normal immunophenotype	Aneuploid with aberrant immunophenotype
Initial Diagnosis	50	27	24	13*

* In one case, two aneuploid populations were detected (15% with a DNA index of 0.66 and 70% with a DNA index of 1.32)

Table 2: Laboratory data from patients with a normal ALL immunophenotype and aneuploid neoplastic cells

				Day 14-28 marrow		Post-remission Status	
	Age/Sex	Initial specimen	DNA Index	% by IP	% aneuploid	% by IP	% aneuploid
1	11/F	Marrow	0.61	ND	ND	<10*	15
2	3/M	Marrow	1.43	0	0	<5*	15
3	35/F	Marrow	1.19	ND	ND	<10*	28
4	10/F	Marrow	1.09	ND	ND	>90	>90
5	71/M	ND	0.79**	ND	ND	40	48
6	9/M	Marrow	1.13	<5	8	RM	RM
7	7/F	Blood	1.24	20	17	RM	RM
8	3/F	Blood	1.14	40	57	RM	RM
9	59/F	Marrow	1.12	0	0	RM	RM
10	2/M	Marrow	1.16	0	0	RM	RM
11	6/F	Marrow	1.17	ND	ND	RM	RM
12	15m/M	Blood	1.27	ND	ND	RM	RM
13	3/M	Marrow	1.17	ND	ND	RM	RM
14	51/F	Marrow	0.76	0	0	RM	RM
15	15/M	Marrow	1.15	0	0	RM	RM
16	7/F	Marrow	1.25	0	0	RM	RM
17	2/F	Marrow	1.18	ND	ND	RM	RM
18	3/F	Marrow	1.21	ND	ND	RM	RM
19	4/F	Marrow	1.19	0	0	RM	RM
20	2/F	Marrow	1.17	ND	ND	RM	RM
21	1/F	Blood	1.35	0	0	RM	RM
22	3/F	Blood	1.14	0	0	RM	RM
23	5/M	Marrow	1.08	ND	ND	RM	RM
24	3/F	Marrow	1.19	ND	ND	RM	RM

*Since the ALL immunophenotype was not unique, these percentages are only estimates and the diagnose could not be determined with confidence by immunophenotype alone

**This value represents the DNA index identified at relapse, no data was available prior to relapse for this patient

ND = No Data available

RM = Remission

Table 3: Laboratory data from patients with an aberrant ALL immunophenotype (CD33+ and/or CD13+) and aneuploid neoplastic cells

				Day 14 marrow		Post-remission status	
	Age/Sex	Initial specimen	DNA Index	% by IP	% aneuploid	% by IP	% aneuploid
1	20/F	Marrow	0.63	0	2	0	4
2	13/F	Blood	0.56	0	5	0	2
3	14/M	Marrow	0.66/1.32*	0	0	57	90
4	3/M	Blood	1.21	0	0	31	52
5	4/M	Marrow	1.22	0	0	51	72
6	14/M	Marrow	1.22	ND	ND	93	96
7	57/M	Blood	1.22	15	20	RM	RM
8	19/M	Blood	1.08	ND	ND	RM	RM
9	7/M	Marrow	1.19	0	0	RM	RM
10	5/F	Marrow	1.21	ND	ND	RM	RM
11	14/M	Blood	1.22	ND	ND	RM	RM
12	4/M	Marrow	1.82	ND	ND	RM	RM
13	6/F	Marrow	1.18	ND	ND	RM	RM

*Two aneuploid populations were detected (15% with a DNA index of 0.66 and 70% with a DNA index of 1.32); only the hyperdiploid population was detected at relapse.
ND = No Data available
RM = Remission

Table 4. Mixing study data

%U937 cells mixed in blood	% determined by DNA ploidy analysis	% determined by modeling program
20.00%	20.50%	20.67%
4.00%	2.72%	3.13%
0.40%	0.22%	Unsuccessful
0.04%	0.07%	Unsuccessful
0.004%	0.06%	Unsuccessful
0%	0.04%	Unsuccessful

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